Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

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General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	[redacted]
1.2	Provide the name of the licenced establishment.	Biomedical Primate Research Centre
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 1 — Type of animal procedure: Long-acting antibiotics in Macaca mulatta: Pharmacokinetics, microbiome and resistome characterization

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The primary objective of this study is to describe the pharmacokinetics (PK) of long-acting antibiotics in Macaca mulatta. In addition, we will analyse the faecal microbiome and resistome before, during and after treatment. All proposed antibiotics will be evaluated and approved by our internal animal welfare body. In addition, along with the proposed antibiotic, we will submit the corresponding details like dosage and timepoints of blood sampling.

We will use long-acting antibiotics registered for veterinary use. Depending on their classification, we will choose the most suitable predictor of efficacy. As an appropriate and efficacious dose for macaques is not yet established, the initial dose will be extrapolated from species in which that specific antibiotic is already approved by allometric scaling.

We consider a dose efficacious when it achieves target values of appropriate PKPD indices for that particular antibiotic (T>MIC or AUC/MIC). The MICs used in these calculations will be based on published data or determined from bacterial isolates acquired during our annual health screening-programme.

We will start with 2 animals for each antibiotic. Prior to administration of the antibiotic we will collect one blood, rectal swab, faeces and urine sample (T=0). After administration of the long-acting antibiotic, blood, urine, and faeces will be collected, and concentrations of antibiotic will be determined to investigate the pharmacokinetics in macaques. The samples will be analysed. Based on the results of these first two

animals, the sampling times may be adjusted for the next two animals to ensure the best possible description of the time-concentration profile. Subsequently, rectal swabs are obtained to analyse gut-microbiome and resistome. These samples will be analysed by genomic sequencing methods. Genomic analyses are more sensitive and therefor preferable over traditional cultering methods[1]. By sampling at different timepoints (before, during and after treatment) it is possible to identify changes in bacterial composition and antibiotic resistant genes under antibiotic pressure.

The blood samples that will be collected with sampling times varying between the different antibiotics and based on the expected plasma concentration-time profile from data in other species. Typically, PK modelling requires a sampling schedule to match the elimination kinetics, more condense initially and ending when the concentration reaches zero. A total of 10 to 12 samples distributed over several days is typical, to be extended in case of repeated dosing to 10 days and several more samples.

The PK data collected from the first two animals will be analysed using a non-linear mixed effects model. This model will then be used to predict the dosage regimen needed to achieve the target PKPD index for that antibiotic. Two additional animals will be treated with this dosage regimen to validate the model. If the predicted dosage is too high or too frequent to be safely or practically administered to macaques, the study will be stopped for that particular antibiotic prior to treating the additional two animals.

Qualified caretakers will perform daily observations for two weeks post administration to specifically document any potential injection site reactions. Additionally, stool quality assessments will be obtained for two weeks post-administration using an objective faecal score to check for possible gastrointestinal side effects.[2,3] These observations will be complemented by routine daily health monitoring throughout the treatment and washout periods for subjective appetite, hydration, and stool quality assessments.

References

1- Gupta, S., Mortensen, M.S., Schjørring, S. *et al.* Amplicon sequencing provides more accurate microbiome information in healthy children compared to culturing. *Commun Biol* **2,** 291 (2019). https://doi.org/10.1038/s42003-019-0540-1

2- Blackwood RS, Tarara RP, Christe KL, Spinner A, Lerche NW. 2008. Effects of the macrolide drug tylosin on chronic diarrhea in rhesus macaques (Macaca mulatta). Comp Med 58:81-87.

3- Mi Young Yoon and Sang Sun Yoon. Disruption of the Gut Ecosystem by Antibiotics. Yonsei Med J. 2018;59: 4–12.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Before administration of the drug, the bodyweight of the animals will be determined to administer the drugs in the right dose. One blood sample (T=0) will be collected prior to administration of the antibiotic. Dosing of the antibiotic will be performed under close surveillance of the veterinarian, while the animal is sedated. The antibiotic injections will be given IM or SC. To simplify the observations of the injection site area, the hairs will be shaved from the overlying skin.

The injection frequency is depending on the halflife of the used antibiotic. We will use the dose interval recommended by the manufacturer as a full treatment and this will be at least one injection and will be maximised at three injections. Only antibiotics with a dosing interval of at least 48 hours will be used. The injected volume will not exceed two ml per injection site, no more than 2 injection sites will be used.

Blood sampling will be done from the vena femoralis while the animal is sedated. Blood samples will be collected at fixed timepoints after administration and are based on the expected plasma concentration-time profile from data in other species. The cumulative blood volume to be taken will not exceed the 1% of the body weight of the animals per month. Every time that the monkey is sedated for a blood sampling, the bodyweight will be recorded and the injection site of the antibiotic will be checked for possible local adverse effects. During the first period of daily blood sampling, the animals will receive tube feeding to prevent dehydration and a negative energy balance.

After administration of the long-acting antibiotic, all the produced urine and faeces will be collected, and concentrations of antibiotic will be determined. In addition rectal swabs are obtaien for microbiome and resistome analysis. Collection both samples is of utmost importance to monitor a) possible development of resistancy in the commensal gutflora, b) the amount of antibiotic entering the environment and c) concentration of the antibiotic reached in the faeces and the bladder. Urine and faecal samples are taken prior to administration and afterwards voided over multiple timepoints.

Urine samples will be collected, filtered, and stored below -20^{0}C until analysed. Faecal samples will be stored below -20^{0}C and below -80^{0}C (microbiome) until analysed. These samples will be collected with a collection tray underneath their home cage. Traditionally animals were housed individually when determining excretion patterns. However, recent research suggests that it is not always necessary to collect individual samples[1,2]. This welfare improvement makes us highly motivated to house the animals in pairs during these PK studies. Because both animals will receive the same dosing and sampling scheme, there is no need for single housing.

Only in the event that this fails during the first trial and we are not able to resolve the problem otherwise, we will house the animals individually for the period required to collect urine and faecal samples. As soon as possible, the animals will be socially pair-housed again.

During the entire study, all animals will be observed daily for general health and for possible local adverse reactions to the injected antibiotic.

1-Hansen, J.J. A novel approach to conducting metabolism studies allowing Non-Human primates to be group housed. Proceedings EPV Seminar 2019, Rome
2- Kendrick J, Stow R, Ibbotson N, et al. A novel welfare and scientific approach to conducting dog metabolism studies allowing dogs to be pair housed [published online ahead of print, 2020 Feb 16]. Lab Anim. 2020;23677220905330. doi:10.1177/0023677220905330

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The goal of these studies are to describe the PK of selected antibiotics in macaques. The number of animals needed to estimate the average value of the PK parameters with an acceptable level of confidence is dependent on the expected magnitude of the inter-individual variability. With a sample of 4 animals, average PK parameter values will be estimated within half a standard deviation (SE = SD/sqrt(n)). Since the animals for this study come from a relatively homogenous population of healthy adults with similar body condition, we can expect based on previous PK studies that the standard deviation will not exceed 20% of the parameter value. With 4 animals, we will therefore be able to estimate the average PK parameters within 10% of the actual value. The obtained data will be fit to a compartmental pharmacokinetic model using nonlinear mixed effects modeling whereby the samples from all the animals will be combined to describe the typical time-concentration profile as well as the inter-individual variability for the sample population of 4 animals.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

The experiment will be performed in clinically healthy, socially housed adult outbred Macaques (*Macaca Mulatta)*. Animals originate from our institute's in-house breeding colony and will remain housed at the property. A complete physical, haematological, and biochemical examination will be performed on all animals prior to the study. Animals will be selected for a uniform nutritional status and body condition score of three.[1,2]

Macaques are extensively used in biomedical research and our institute houses a big breeding colony (n=600). As there is a lack of information regarding efficacy of long-acting antibiotics in monkeys, we choose Macaca mulatta as target species.

To test several long-acting antibiotics in a five-year period we request a maximum of 20 resus macaques.

As there are no sex differences recorded in swine and cattle, we don't have sex preference. Adult animals are requested.

References
1- Clingerman KJ, Summers L. 2005. Development of a body condition scoring system for nonhuman primates using Macaca mulatta as a model. Lab Anim (NY) 34:31–36.
2- Clingerman KJ, Summers L. 2012 Validation of a body condition scoring system in rhesus macaques (Macaca mulatta): inter- and intrarater variability J Am Assoc Lab Anim Sci. 51:31-36.

C. Re-use

Will the animals be re-used?

☐ No, continue with question D.

x Yes > Explain why re-use is considered acceptable for this animal procedure.

- Animals that will be used in this experiment have possibly been used in previous experiments. Their cumulative discomfort will be taken into account. The expected discomfort in this study is moderate. Due the long life expectancy of macaques, the animals are returned to the experimental stock after this study.
- The limitations described in art 1e of the Wet op de Dierproeven will be applied.

Are the previous or proposed animal procedures classified as 'severe'?

x No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement
The body is very complex and the *in vivo* interactions are not completely understood. At present there is no *in vitro* model available that can mimic the (macaque) body system sufficiently. Physiologically-based pharmacokinetic (PBPK) modelling is an *in silico* method used in toxicology and risk assessment to predict the kinetics of compounds in a new species. PBPK models often predict the pharmacokinetics of new compounds with an error that can be up to an order of magnitude. This makes PBPK models suitable for risk assessment and the determination of initial doses to be used for *in vivo* studies, but not for designing effective clinical dosage regimens in target animal species. I*n vivo* studies describing the pharmacokinetics of a compound in the target animal remain the gold standard for dosage regimen design. To the authors' knowledge, there have been no pharmacokinetic studies of long-acting antibiotics in macaques. The analyses of the complex microbiome and resistome also requires live donor animals because the complex microflora cannot be maintained ex vivo.
Determination of MIC_{90} values are in vitro procedures and will be performed prior to the start of the study or will be extrapolated when the sensitivity of bacteria to the selected antibiotics have already extensively been studied.

Reduction
The PK data collected from the first two animals will be analysed using a non-linear mixed effects model. This model will then be used to predict the dosage regimen needed to achieve the target PK/PD index for that antibiotic. Two additional animals will be treated with this dosage regimen to validate the model. If the predicted dose is too high to be safely or practically administered to macaques, the study will be stopped for that particular antibiotic prior to treating the additional two animals. Regardless of the outcome, we intend to publish this data.

Refinement
Animals are trained to cooperate as much as possible for the procedures such as receiving sedation. More important, this study itself will contribute to refinement. A positive outcome can reduce stress caused by daily-dose treatment schedules.

References
1- Pelkonen O, Turpeinen M, Raunio H. In vivo-in vitro-in silico pharmacokinetic modelling in drug development: current status and future directions. *Clin Pharmacokinet*. 2011;50(8):483-491. doi:10.2165/11592400-000000000-00000

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

Because the animals are under sedation after antibiotic administration for multiple blood samples in a relatively short period of time, the animals will receive tube feeding on that day to ensure they will not suffer from dehydration and a negative energy balance.

Animals will be housed with a socially compatible animal. There is an extensive program for enrichment in our institute that consists of playing material and methods to present food.

During the study, animals will be observed daily by qualified animal caretakers for general health and for possible adverse reactions to the injected antibiotic. Should changes occur in behaviour, appetite or stool a veterinarian will be consulted and measures will be discussed with the investigator and implemented. Possible local reactions on the injection site of the antibiotic will be recorded at multiple time points using a scoring system that includes redness, swelling and induration.

No adverse effects to the environment are expected.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

Not applicable

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

No

x☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Only when pair housing cannot be maintained the animals will be temporarely single housed during 168h (depending on tested antibiotic) and the period of time will be as short as possible. The animals will be housed in a way that they can see each other. Single housing could be neccesary to obtain faecal en urine samples to study clearance pathways of the antibiotic in macaques.

G. Location where the animals procedures are performed

Ẅill the animal procedures be carried out in an establishment that is not licenced by the NVWA?

x No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

No > Continue with question I.

xYes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

Depending on the tested long-acting antibiotic tested, there is a possibility that the animals are experiencing pain after the procedure. For example, in dogs[1] and pigs[2] injection site pain has been described after oxytetracycline injections. In contrast, we used Tulathromycin off-label in some animals with multi-resistant bacterial infections and we did not see any adverse effects during treatment. However, swelling and redness of the injection site and/or intestinal dysbacteriosis might occur in any of the tested substances.

1-D.A.Y. Adawa, A.Z. Hassan S.U. Abdullah , A.B. Ogunkoya , J.B. Adeyanju & J.E. Okoro (1992) Clinical trial of long-acting oxytetracycline and piroxicam in the treatment of canine ehrlichosis, Veterinary Quarterly, 14:3, 118-120, DOI: 10.1080/01652176.1992.9694345
2- XIA, W. GYRD-HANSEN, N. and NIELSEN, P. (1983), Comparison of pharmacokinetic parameters for two oxytetracycline preparations in pigs. Journal of Veterinary Pharmacology and Therapeutics, 6: 113-120. doi:10.1111/j.1365-2885.1983.tb00387.x

x☐ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

In case of a painful injection site, NSAID's will be administered. As mentioned before, the animals will be observed daily by qualified animal caretakers for general health and for possible adverse reactions to the injected antibiotic.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

- Possible adverse effects of antibiotics. However, generally not serious and they occur at a low frequency.
- Repeated sedation for blood sampling
- (Separation from its buddy for 24 hr urine and faeces collection, only as a last resort solution)

Explain why these effects may emerge.

- Systemic side effects of the injected antibiotics can be caused by individual hypersensitivity to a substance in the formulation (allergic reaction), local side effects can occur due to the nature of the formulation (components for slow release).
- Sedation can cause nausea and a temporarily decreased appetite
- (Conflicts after re-introduction in case pairhousing couldn't be maintained)

Indicate which measures will be adopted to prevent occurrence or minimise severity.

- Qualified caretakers will perform daily observations for two weeks post administration to specifically document any potential injection site reactions. The injections will be performed *lege artis*.
- Additionally, stool quality assessments will be obtained for two weeks post-administration using an objective faecal score to check for possible gastrointestinal side effects. These observations will be complimented by routine daily health monitoring throughout the treatment and washout periods for subjective appetite, hydration, and stool quality assessments.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

X No > Continue with question K.

☐ Yes > Describe the criteria that will be used to identify the humane endpoints.

Indicate the likely incidence.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

IM and SC dosing under sedation: mild
Repeated blood sampling under sedation: moderate
Only when we have no other option:
Separation from its buddy for urine and faeces collection: moderate
The total amount of discomfort is estimated as moderate.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

x No

☐ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☐ Yes